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Optimization of Dosage Regimen of Gentamicin against Pasteurella multocida in Bovines: Old Drug, New Approach

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Abstract

Background: Gentamicin is commonly used to treat Haemorrhagic Septicemia (HS) and other respiratory tract infections in bovines. But no data on its doses and schedules optimized for therapeutic success against *P. multocida* using pharmacokinetics (PK) - pharmacodynamic (PD) integration and modeling is available in farm animals. We investigated PD of gentamicin against *P. multocida* isolates and optimized the dosage schedules in buffalo species using novel approach of PK-PD modeling.

Results: The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of gentamicin against *P. multocida* in Mueller Hinton Broth (MHB) and serum were similar. The MBC:MIC ratios were 1.23 and 1.27, respectively, for MHB and calf serum.. The mutant prevention concentration (MPC) =6 µg/mL of gentamicin was higher than MIC (0.75 µg/mL) and MBC (0.95 µg/mL). *In vitro* growth inhibition curves of gentamicin in MHB and serum were characteristic of bactericidal activity against *P. multocida*. Time kill kinetics demonstrated that killing activity of gentamicin is concentration andtime dependent.. The values of PK-PD indicators, C_{max}/MIC and AUC_{24h}/MIC were 21 and 61 h, respectively. Based on MPC, PK-PD indices, C_{max}/MPC and AUC_{24h}/MPC were 2.60 and 7.62 h, respectively. Using PK-PD modelling, the predicted gentamicin values of AUC_{24h}/MIC for bacteriostatic bactericidal action and bacterial eradication were 32.13, 47.15 and 60.96 h, respectively.

Conclusions: The PK-PD indicespredicted therapeutic success of the gentamicin against *P. multocida*. Based on PK-PD modeling, optimum daily dosage of gentamicin was 2-2.5 mg/kg for treating infections caused by *P. multocida* (MIC_{90} ≤1.0 µg/mL) in buffalo calves. However, in difficult clinical infections associated with pathogens of MIC_{90}≤4.0 µg/mL, a higher dosage of 7.5 mg/kg is recommended. Low MPC of gentamicin against *P. multocida* suggested low selection pressure for emergence and amplification of resistant subpopulation during treatment.

Keywords: Gentamicin; *Pasteurella multocida;* Time-kill kinetics; Mutant Prevention Concentration; PK-PD integration; PK-PD modeling

Background

The development of antimicrobial resistance from the extensive use of antimicrobial agents and a decline in the development of new agents is threatening human and animal health, and once again infections are becoming untreatable due to emergence of multidrug resistant bacteria. Appropriate use of antibiotics is advocated because there is strong evidence that the dose administered of an antimicrobial to an animal can influence the likelihood of emergence of resistance [1-3]. Therefore, designing optimal dosage schedules of antimicrobials is critical to achieve therapeutic success and prevention of emergence of resistance. For this a comprehensive study on pharmacokinetics (PK) and pharmacodynamics (PD) of antimicrobials in target species is recommended [4,5]. Pharmacokinetic models describe the relationship between the dose of a drug and resulting concentrations at different sites of the body. Pharmacodynamic models describe the relationship between drug concentrations in plasma/serum or tissues and resulting therapeutic effect. Improving dosing strategies for antimicrobial drugs that are already clinically available is an effort to increase the life span of antibacterial drugs to treat the infectious diseases. PK-PD modeling provides a quantitative description of the time course of drug disposition and drug effects and dose optimization of antimicrobial agents by this method had been suggested by various workers [5-7].

The Haemorrhagic Septicaemia (HS) caused by *Pasteurella (P.) multocida* is of great socio-economic importance across South and Southeast Asia, Africa and many other regions of the world. It is an acute and fatal disease primarily occurring in cattle, water buffaloes and camels, and occasionally in other domesticated and wild mammals [8]. In Asia, buffalo is the most important dairy animal as 95% of milk produced is from buffaloes. The HS results in severe economic losses and is ranked as the most important contagious disease of cattle and buffaloes with young animals being most at risk of successive outbreaks [8,9]. Gentamicin, a broad spectrum aminoglycoside has been widely used for the therapy of serious respiratory tract infections including HS associated with *Mannheimia haemolytica* and *P. multocida* in buffaloes, cattle, goats, pigs, sheep, and rabbits. Gentamicin has a narrow therapeutic index (minor difference between toxic and therapeutic blood concentration). Despite the risk of toxicity development, it has the advantage of high clinical efficacy in serious infections and low cost compared to newer agents licensed to use in veterinary medicine. Therefore, the importance of dose optimization of gentamicin for therapy in target (buffalo) species cannot be overlooked. Gentamicin

Citation: Sidhu PK, Kaur G, Kumbhar G, Balaje RM, Gupta MP (2014) Optimization of Dosage Regimen of Gentamicin against *Pasteurella multocida* in Bovines: Old Drug, New Approach. J Veterinar Sci Technol 5: 199. doi:10.4172/2157-7579.1000199

was developed several decades ago and dosage schedules used for this drug are not based on the modern drug development programs.

Earlier, the dose of antimicrobials was selected so that the plasma concentration of the drug exceeded the minimum inhibitory concentration (MIC) for as long as possible. In the last decades, studies using *in vitro* and animal models have played a significant role in obtaining a more detailed understanding regarding the relationship between the PK and PD properties of antibacterial agents. Clinical success of antimicrobials have been correlated with bacterial killing using surrogate markers derived from PK and PD data employing techniques of PK-PD integration and/or PK-PD modeling [4,6,7]. Recently, determination of PK-PD indices using mutant prevention concentration (MPC) viz., area under serum concentration versus time curve (AUC)/ MPC and peak drug concentration (C_{max})/MPC ratio have been proposed to restrict the emergence and enrichment of resistance mutants [10,11]. Therefore, there is need for modeling dosing regimens using MPC-based thresholds to restrict emergence of resistance and it requires generation of databases in which MPC is determined for clinical isolates of target pathogen. The study was undertaken to conduct PD, PK-PD integration and PK-PD modeling of the gentamicin data for dose optimization to ensure clinical success and minimization of the selection of mutants with the objectives: (i) to measure the MIC, minimum bactericidal concentration (MBC) and MPC of gentamicin against *P. multocida*; (ii) to determine the time-kill kinetics of gentamicin for *P. multocida* over an incubation period of 24 h; (iii) to obtain PK-PD indices by integrating PK-PD data for achieving optimum therapeutic success of gentamicin against HS in buffalo species; (iv) to establish MPC based PK-PD indices predicting the emergence of resistance for gentamicin against *P. multocida*; (v) to design the rational dosage schedule of gentamicin against *P. multocida* in buffalo species using PK-PD modeling approach

Methods

Drugs and organisms used

A pure standard of gentamicin was purchased from M.P. Biomedicals India, Pvt. Ltd. Mumbai. A pathogenic strain of *P. multocida* (P52:B2) was supplied by the Punjab Veterinary Vaccine Institute, Ludhiana, India. Another strain of *P. multocida* (serotype unknown) was obtained from a buffalo suffering from HS. The fresh culture was grown on blood agar (BA) plates by incubating the plates statically at 37°C.

Determination of MIC and MBC

For gentamicin, MIC and MBC were determined *in vitro* in MHB and serum of buffalo calves for two isolates of *P. multocida*. MIC and MBC were determined by Micro-dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [12]. Five overlapping sets of doubling dilutions were used to increase the accuracy. Briefly, nine to ten colonies were used to inoculate 10 mL of MHB/ serum, followed by incubation at 37°C on a shaking incubator (220 rpm) for 5 h and final cell count were approximately $2-3 \times 10^8$ cfu/mL. A 10 μL ($\sim 2-3 \times 10^6$ cfu/mL) of this culture was used to inoculate each well of multiwell plate having broth or serum samples with known concentrations of gentamicin. Getamicin solution (0.20 mL; in MHB/serum) at twice the required final maximum concentration was added to 0.20 mL of MHB and serum as described by Sidhu et al. [13]. Bacterial counts expressed as cfu/mL were determined by performing spot plate counts on BA plates following serial dilutions in phosphate buffer saline. The serial dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were prepared. Four 25 μL drops of the appropriate dilutions were dropped on the BA plates, allowed to dry and incubated at 37°C for 18

h. Bacterial counts were determined for each 25 μL drop and lower limit of quantification was 10 cfu/mL. A MBC was determined in accordance with CLSI guidelines [12] using a single set of doubling dilutions. The MIC well and three other wells with drug concentrations higher than MIC were used to establish MBC using the spot plate count method. The lowest drug concentration which completely inhibits the growth of bacteria on plates after 18 h incubation was recorded as MBC.

Determination of MPC

The MPC of gentamicin was determined in MHB following the previously described method by Blondeau and co-workers [11]. Briefly, a natural isolate of *P. multocida* was freshly grown from stocks stored at -20°C for each experiment. A starter culture was prepared by inoculating 400 mL of MHB with all the single colonies of the strain obtained on BA plate and then incubating overnight at 37°C in static incubator. Next morning, this culture yielded $\sim 1-2 \times 10^{10}$ cfu/mL. One ml of this culture was diluted (1:10) in pre-warmed MHB and put on orbital shaking incubator for 6 h at 37°C to provide an estimated growth of $6-7 \times 10^{10}$ cfu/mL. A 100 μL of this culture was used to inoculate already prepared BA plates having different concentrations of gentamicin and plates were incubated at 37°C with 0.1% carbon dioxide. The drug concentrations used were: 0×MIC, 1×MIC, 2×MIC, 3×MIC, 4×MIC, 5×MIC, 6×MIC, 8×MIC, 10×MIC, 12×MIC, 16×MIC, 20×MIC, 24×MIC, 32×MIC, 48×MIC, 64×MIC, 96×MIC μg/mL. The plates were incubated for 72 h and were checked for growth of the pathogens after every 24 h. The MPC was recorded as the lowest multiples of MIC that prevented the growth of bacteria completely at 72 h of incubation. The each experiment was done in triplicate on different days to take into account the inter-day and intra-day variability. The initial inoculums size of the culture was determined with each experiment by serial dilutions (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) and spot-plate counts.

Time kill kinetics

In vitro time-kill curves of gentamicin were established on MHB and buffalo calf serum as described by Aliabadi and Lees [14]. Briefly, a natural isolate of *P. multocida* was freshly grown from stocks stored at -20°C for each experiment. A starter culture was prepared by inoculating 10 mL of MHB with 5-6 colonies of the strain to be tested and then incubating overnight at 37°C in an orbital shaking incubator to provide an estimated growth of 1×10^8 cfu/mL. Fifty μL of this culture were diluted 1:50 in freshly prepared pre-warmed MHB/serum and incubated statically for 1 h at 37°C. The cells were centrifuged at 3100 g for 2 min. The supernatant was discarded and the cells re-suspended in 50 μL phosphate buffered saline. The counts of viable cells ($1-2 \times 10^7$ cfu/mL) were determined by serial dilution and spot-plate counts. Volumes of 390 μL MHB and serum were pre-warmed in an incubator at 37°C and each was inoculated with 10 μL of the starter culture to give a final volume of 400 μL. The cultures were then placed in an orbital shaking incubator at 37°C. Four drops of 25 μL of each culture was sampled and the viable count was determined by serial dilution and spot-plate counts after 0, 1, 2, 5, 8, 10 and 24 h incubation. The lower limit of quantification was 10 cfu/mL.

Determination of PK-PD indices

The mean values of PK-PD indices correlating bacteriological outcome, C_{max}/MIC and AUC_{24h}/MIC were calculated using published PK data of gentamicin in buffalo species [15] for serum against *P. multocida* (Table 2). In addition, PK-PD variables, C_{max}/MPC and AUC_{24h}/MPC were calculated to determine the values required to prevent the selection of *P. multocida* mutants against gentamicin.

PK-PD modeling

For PK-PD modelling, PD parameters were calculated using the nonlinear Win-Nonlin regression programme (Pharsight Corporation, Mountain View, CA, USA). The AUC_{24h}/MIC data from *in vitro* bacterial growth inhibition curves were modelled to the Sigmoidal E-max equation (1):

$$\text{Dose} = \frac{CL \times \left(\frac{AUC_{24h}}{MIC} \right) \times MIC_{90}}{F} \qquad (1)$$

where E_o is the change in log cfu/mL of sample (serum) after 24 h incubation in the control sample (without gentamicin) compared to the initial inoculum log cfu/mL, Emax is the maximum antibacterial effect determined as difference in log cfu/mL in samples incubated with gentamicin between time 0 and 24 h when the detection limit (10 cfu/mL) is reached, C_e is the AUC_{24h}/MIC in the effect compartment (*ex vivo* site), and N is the Hill coefficient which describes the steepness of the AUC_{24h}/MIC-effect curve.

The antibacterial effect of gentamicin was quantified for three levels of growth inhibition by calculation of AUC_{24h}/MIC for bacteriostatic action, bactericidal action and bacterial eradication. AUC_{24h}/MIC values for bacteriostatic and bactericidal actions were defined as those values which produced E=0 (no change in bacterial count after 24 h incubation) and AUC_{24h}/MIC=-3 (a 3 log or 99.9% reduction of the original inoculum count after 24 h incubation), respectively. For bacterial eradication AUC_{24h}/MIC was defined as the lowest AUC_{24h}/MIC which provided a 4 log reduction in bacterial count of original inoculums.

Results

Determination of MIC, MBC and MPC

The gentamicin MICs, MBCs and MPCs for the two strains of *P. multocida* in Mueller Hinton Broth (MHB) and serum are presented in Table 1. The MICs and MBCs of gentamicin in MHB were not much different than the values obtained in serum. The MBC: MIC ratios ranged from 1.23 to 1.27 for both fluids. The MPC (MPC=8×MIC) was higher than MIC and MBC (Table 1).
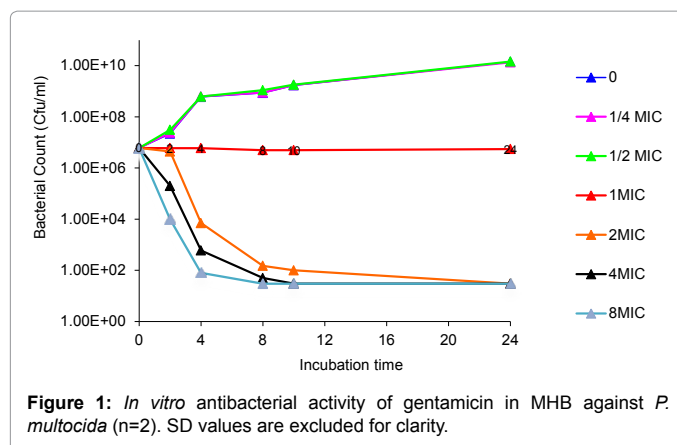
In vitro growth inhibition curves

In vitro growth inhibition curves of gentamicin in MHB and serum against *P. multocida* are illustrated in Figures 1 and 2. Values are mean of two strains, standard error bars are not shown for clarity. The growth inhibition curves were characteristic of a concentration and time-dependent killing action of gentamicin. The bacterial count increased by > 100-foldsafter 24 h incubation in control samples. The viable count decreased by 3-log colony forming units (cfu)/mL after 2 h of incubation at 4×MIC for isolates of *P. multocida* and reduction of 4-log cfu/ml occurred at 5 h of incubation at 2,4 and 8×MIC. There was
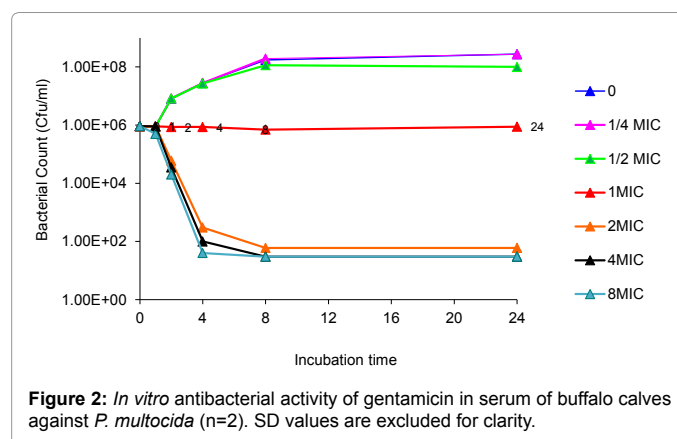

Figure 1: *In vitro* antibacterial activity of gentamicin in MHB against *P. multocida* (n=2). SD values are excluded for clarity.

Figure 2: *In vitro* antibacterial activity of gentamicin in serum of buffalo calves against *P. multocida* (n=2). SD values are excluded for clarity.

Parameter	Unit	Mean ± SD
C_{max}	μg/mL	15.89 ± 0.85
AUC_{0-24h}	μg.h/mL	45.90 ± 0.30
$MIC_{(serum)}$	μg/mL	0.75 ± 0.00
$MBC_{(serum)}$	μg/mL	0.95 ± 0.05
MPC	μg/mL	6.0 ± 0.00
C_{max}/ MIC	-	21.00 ± 0.80
AUC_{0-24h}/ MIC	h	61.00 ± 0.30
C_{max}/ MBC	-	17.00 ± 0.80
AUC_{0-24h}/ MBC	h	48.00 ± 0.40
C_{max}/ MPC	-	2.60 ± 0.80
AUC_{0-24h}/ MPC	h	7.62 ± 0.30

C_{max}: peak concentration; AUC: area under the curve; C_{max}/MIC = ratio of *in vivo* C_{max} to *in vitro* MIC; $AUC_{0-24 h}$/MIC: ratio of *in vivo* AUC for 24 h to *in vitro* MIC; C_{max}/ MBC: ratio of *in vivo* C_{max} to *in vitro* MBC; $AUC_{0-24 h}$/MBC: ratio of *in vivo* AUC for 24 h to *in vitro* MBC; C_{max}/MPC: ratio of *in vivo* C_{max} to *in vitro* MPC; $AUC_{0-24 h}$/MPC: ratio of *in vivo* AUC 24 h to *in vitro* MPC.
*PK data was taken from work of Baxla et al. [20].

Table 2: Integration of *PK-PD data obtained from buffalo calves (n=5) administered with gentamicin by intravenous route (5 mg/kg).

complete eradication at 8 h and 24 h of incubation at concentrations of 4 and 8×MIC. A high level of kill was attained with 8-24 h of exposure and the concentration-response relationship was steep. Moreover, there was no re-growth when organisms were exposed to gentamicin concentrations of 8×MIC for 8 h or more.

PK-PD integration

The PK-PD indices obtained by integrating *in vivo* PK data with *in vitro* PD data of gentamicin for twod strains of *P. multocida* are

Parameter	Matrix	Strain 1	Strain 2	Mean ± SD
MIC	MHB	0.75	0.8	0.78 ± 0.02
	Serum	0.75	0.75	0.75 ± 0.00
MBC	MHB	0.9	1	0.95 ± 0.05
	Serum	0.9	1	0.95 ± 0.05
MBC/MIC	MHB	1.2	1.25	1.23 ± 0.02
	Serum	1.2	1.33	1.27 ± 0.06
MPC	MHB	6	6	6.00 ± 0.00
MPC/MIC	MHB	8	8	8.00 ± 0.00

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; MPC: mutant prevention concentration.

Table 1: MIC, MBC and MPC (μg/mL) of gentamicin against *P. multocida (n=2)*.

Parameter	Unit	Mean ± SD
Log E_0	Cfu/mL	2.39 ± 0.09
Log E_{max}	Cfu/mL	-4.51 ± 0.11
Log E_{max} - E_0	Cfu/mL	-6.91 ± 0.16
AUC_{24h}/MIC for bacteriostatic action	h	32.13 ± 2.05
AUC_{24h}/MIC EC_{50}	h	36.24 ± 1.01
AUC_{24h}/MIC for bactericidal action	h	47.15 ± 4.72
AUC_{24h}/MIC for bacterial eradication	h	60.96 ± 3.85
Slope (N)	-	4.859 ± 0.71

E_0: difference in bacterial count in control sample (without drug) between time 0 and 24 h; E_{max}: difference in bacterial count in sample incubated with gentamicin between time 0 and 24 h, when the detection limit is reached; AUC_{24h}/MIC EC_{50}: AUC_{24h}/MIC of drug producing 50% of the maximum antibacterial effect; N: slope of the AUC_{24h}/MIC - response curve.

Table 3: PK– PD modeling of gentamicin against *P. multocida* using *in vitro* serum data in buffalo calves.

given in Table 2. The C_{max}/MIC was 21 (MIC= 0.75 µg/mL in MHB and serum) which exceeded the recommended literature value of 8-10. Another PK-PD indicator, AUC_{24h} /MIC (61 h) for treatment efficacy was below the target of 80 h. The PK-PD indices correlating the risk of emergence of resistance, AUC_{24h} /MPC and C_{max}/MPC were 7.62 h and 2.60, respectively (Table 2).

PK-PD modeling

PK-PD modelled data for gentamicin are presented in Table 3. The predicted AUC_{24h}/MIC values for bacteriostatic, bactericidal and bacterial eradication action were 32.13, 47.15 and 60.96 h, respectively.. Exposure levels were not very high for bactericidal and eradication activity of the drug. The concentration-effect relationship was steep as indicated by the value of Hill-coefficient (N=4.89). The data indicated that gentamicin requires 1.33, 2.0 and 2.5 multiples of MIC for bacteriostatic, bactericidal and eradication activity (Table 3).

Discussion

PK-PD relationships

Recently optimal dosage regimens of antimicrobials are redefined as the dosage schedules which not only maximize the clinical efficacy, but also minimize the selection and spread of microbial resistance [1,16]. PK-PD indices (C_{max}/ MIC, AUC_{24h}/ MIC, percentage of dosage interval for which serum drug concentration exceeds MIC, T> MIC) specific to the group of antibiotics have been recommended as predictors of antibiotic efficacy [1,5,17-20]. For aminoglycosides, bacterial eradication is concentration-dependent, and the therapeutic goal is to maximize drug exposure or these agents are most effective when they have a high C_{max}/MIC ratio [16]. The C_{max}/MIC obtained for gentamicin against *P. multocida* was 20 that exceeded the recommended value of 8-10 (Table 2). AC_{max}/MIC ratio (8) of aminoglycosides within the first 24 h of treatment resulted in around 90% bacteriological as well as clinical cure [21,22]. However, the outcome of antimicrobial therapy depends on a wide range of factors, including disease severity/pathogen load, strain of pathogen and immunocompetence of the patient. Moreover, the killing mechanisms and the amount of drug required may be both 'bug and drug' specific [23]. Therefore, it necessitates that the optimal numerical value of given PK-PD index, such as C_{max}/MIC, should ideally be determined for each drug against a target pathogen. The value of C_{max}/MIC=21 for *P. multocida*, indicates that the drug should achieve clinical cure against the pathogens having MIC ≤0.75 µg/mL when administered at the dose rate of 5 mg /kg body weight by intravenous route [15]. The higher value of C_{max}/

MIC in this study might be due to usage of plasma concentration at zero time (CP^0) instead of C_{max} obtained from previously published study [24]. Nevertheless, gentamicin is likely to achieve C_{max}/MIC=10 or above when administered by intramuscular route because of its good bioavailability (≥ 90%) [24,25]. An AUC_{24h}/ MIC ratio= 61 h of gentamicin fell below the required value of 80 h [20]. It has been reported in man and mice that AUC_{24h}/MIC ratio correlates with cure when C_{max}/MIC ratio was less than 8 and it doesn't relate well when latter ratio is ≥10 [26]. Accordingly, in current study C_{max}/MIC is considered as a better predictor of the gentamicin efficacy and suggested clinical cure of HS in buffalo calves. The results obtained were in simulation with previous work where therapeutic efficacy of gentamicin correlated better with C_{max}/MIC in patients treated for gram negative bacillary infection [27,28]. Low AUC_{24h} /MIC of gentamicin should not be considered as having negative impact on the treatment outcome. Human clinical trials had suggested that therapy with aminoglycosides should be aimed to achieve maximum Cmax/MIC to obtain clinical efficacy and preventing the risk of resistance emergence [29].

There is uncertainty about optimal PK-PD indices of aminoglycosides defining their efficacy and propensity to select resistant mutants. Therefore, it is important for aminoglycosides to determine relationship between serum/plasma concentrations, target pathogen sensitivity, therapeutic response and capacity to prevent the emergence of resistance. A MIC, which is used as basis for dosage regimen does not provide information on the time to kill, the time to maximum kill, the log change within a fixed time, or the maximum reduction in viable counts of bacteria [30]. Time kill kinetic studies address this issue by allowing measurement of the antimicrobial effect of drug quantitatively along with time course over an incubation period of 24 h. Brown and Riviere [22] have proposed that maximum aminoglycoside concentrations at the site of infection should be ≥ 8-10 times the *in vitro* determined MIC in order to minimize resistance development. *In vitro* time-kill studies exhibited concentration and time dependent activity of gentamicin against *P. multocida* in MHB and serum of calves. The *in vitro* time kill data demonstrated virtual eradication of *P. multocida* by gentamicin (reduced colony forming units (cfu)/mL to <10) at concentrations 4 and 8×MIC. Moreover, a bactericidal level of action (3 log reduction in bacterial count) was well maintained up to 24 h after 2 h of incubation both in MHB and serum of buffalo calves. There was no re-growth when organisms were exposed to low gentamicin concentrations *in vitro*. The data generated on time kill kinetics confirmed that the gentamicin is bactericidal against *P. multocida* as described in standard texts for aminoglycosides as a group. Further, there can be marked variations in the killing activity of a single drug against different strains of pathogens belonging to same species and MIC provides no information about it. In fact, strains variation within a bacterial species impact the PK/PD indices, and that such variability needed to be considered when targeting specific therapeutic outcomes [31]. Use of minimum bactericidal concentration (MBC) has been suggested in addition to (or in lieu of) MIC values as a PD parameter to handle the strain differences within the same species of pathogens [32]. The MIC of gentamicin fell within the earlier reported range (0.03-8 µg/mL) against *P. multocida* isolates (n=154) recovered from cattle suffering from upper respiratory tract infections [33]. The MIC_{50} and MIC_{90} of gentamicin determined in this study were 2 and 4 µg/ml, respectively. The lower MIC and MBC obtained in the current investigation than previous reported MIC_{50} indicated the sensitivity of pathogen and utility of gentamicin for the treatment of HS. The clinicians recommend the use of aminoglycosides for treatment in infections associated with pathogens having MIC ≤1 µg/ml [34]. Predicted daily doses of gentamicin were calculated using equation 2 (Table 4).

*MIC_{90}	Bacteriostatic[a]	Bactericidal[b]	Eradication[c]	**AUC_{24h}/MIC = 80
1	1	1.46	1.9	2.48
2	2	2.91	3.8	4.96
4	4	5.82	7.6	9.92

*MIC_{90}=minimum inhibitory concentration (μg/mL) for 90% isolates; AUC_{24h}/MIC: ratio of *in vivo* $AUC_{(0-24h)}$ to *in vitro* MIC.
[a,b,c]Modelled AUC_{24h}/MIC values for bacteriostatic activity (32 h), for bactericidal activity (47 h) and for bacterial eradication (4 log inhibition) = 61 h;
** Recommended AUC_{24h}/MIC value for clinical efficacy of aminoglycosides.

Table 4: Estimated daily dosage (mg/kg) of gentamicin for intravenous administration in buffalo calves.

$$Dose = \frac{CLx\left(\dfrac{AUC_{24h}}{MIC}\right)xMIC_{90}}{F} \qquad (2)$$

Based on a requirement to provide a bactericidal effect, the estimated dose of gentamicin is 2.48 mg/kg against *P. multocida* in buffalo calves to be repeated after every 24 h, with values of Cl, AUC_{24h}/MIC and MBC of 0.031 mL/kg/h, 80 h, and 1.0 μg/mL, respectively. In this study F is not applicable as the drug was administered by intravenous route. The PK-PD relationships suggested that the daily dosage of 2.5 mg/kg body weight would be able to produce clinical and bacteriological cure in buffalo calves against susceptible pathogens having MIC≤ 1.0 μg /mL.

However, several studies have supported that C_{max}/MIC and AUC_{24h}/MIC ratios are predictors of therapeutic outcomes but don't correlate well with the resistance emergence of antimicrobials [11,32,35]. Recently, use of MPC based PK-PD indices have been recommended to restrict the emergence and amplification of mutant subpopulation [10,32,35]. During severe infections, the initial inoculums may exist as a mixture of more than one population of microbes having high and low sensitivity to antimicrobials. An overgrowth of a pre-existing subpopulation with an initially lower level of susceptibility may occur due to exposure to antibiotics and resistance may emerge [36,37]. Therefore, a strategy to reduce the size of the Mutant Selection Window (MSW) that covers the concentration range between the MIC of a wild bacterial population and MPC has been proposed by adjusting the dosage regimen [38]. The therapeutic usefulness of MPC is dependent on obtaining its value below the attainable serum and tissue concentrations of drug post administration at doses that are safe for patients [11].

We integrated our *in vitro* MPC data with published *in vivo* PK information of gentamicin determined in the buffalo calves. Extrapolating from the *in vitro* MPC data indicated that *in vivo* it should be possible to eradicate less susceptible strains of *P. multocida* bearing in mind the MPC (6.0 μg/mL) of gentamicin, although it is possible that mutants could be selected when drug levels in serum/tissues fall in MSW. It seems, eradication of strains that already had some resistance will not be difficult, in view of the higher peak serum concentrations (15 μg/mL) of gentamicin *in vivo*. The C_{max}/MPC and the AUC/MPC ratios were 2.6 and 7.6 h, respectively, for gentamicn against *P. multocida*. The C_{max}/MPC ratio is quite small compared to C_{max}/MIC achieved in the study. However, the value of C_{max}/MPC required for prevention of resistance emergence of gentamicin against *P. multocida* is not known and would be point of interest for future studies.

The studies establishing the MPC/MIC ratios of fluoroquinolones have been documented by many research groups but there is limited information for aminoglycosides on this PD parameter [39-41]. A MPC range of 2-512 μg/mL with MPC_{50} of 44.2 μg/mL against *E.*

coli isolates from human patients have been reported for gentamicin in Nigeria [41]. Compared to this study, although the MPC (6 μg/mL) of gentamicin was lower but MPC/MIC ratio (8) was higher than the reported value of 2.8 by these authors. Low MPC/MIC ratio obtained in human study is due to the fact that MIC_{50} (16 μg/mL) was quite high compared to MIC= 0.75 μg/mL of our data. Moreover, it was not possible for us to determine MIC_{50} because of small number of isolates. However, gentamicin MPC/MIC ratio (8) obtained by us was exactly the same as determined by Miller and co-authors against *E. coli* [42]. The limitation for comparing the results is MPC and MIC values are specific to bacterial species. The MPC/MIC ratios reported for ciprofloxacin were; 24 for *Mycobacterium smegmatis,* 53 for *M. tuberculosis* and 13 for *Staphylococcus aureus* [39]. To the best of our knowledge, no record of the MPC data of gentamicin for *Pasteurella* spp. was traced to compare our data. There is need to establish data on MPC of gentamicin for many isolates of different bacterial species and strains to attain useful information for predicting dosage regimens that will restrict the development of resistance in clinical conditions.

PK-PD modeling

Another approach for predicting efficacy of antimicrobials is use of PK-PD modeling which combines the PK and PD information and describes the time course of antibiotic effect as a function of the drug dosage regimen. The advantage of using PK-PD modeling approach for the selection of dosage regimens of antibiotics to improve the probability of therapeutic success and its potential for minimization of resistance development has been suggested by previous authors [1,7,17]. For antimicrobials it has been emphasized that actual ratios required for providing differing levels of growth inhibition for individual drugs acting against specific pathogens should be used as an alternative to using the widely quoted ratios of C_{max}/MIC and AUC_{24h}/MIC [1,13,32].

The PK-PD modeling approach was applied to gentamicin against two pathogenic strains of *P. multocida* using PK data from buffalo calves which had received a clinically recommended dose of 5 mg/kg body weight. Based on exposure over a 24 h period, AUC_{24h}/MIC values required for three levels of growth inhibition, bacteriostatic and 3 or 4 log reductions in bacterial count were determined. The numerically closer values of AUC_{24h}/MIC were required to achieve both bacteriostatic and bactericidal activity against *P. multocida* with gentamicin. This supports the existing knowledge and present findings of *in vitro* time kill kinetics that gentamicin is bactericidal in nature against *P. multocida*. The modelled AUC_{24h}/MIC ratios providing a 3 log and 4 log reductions in bacterial count with gentamicin were 47 h and 61 h, respectively. PK-PD modeled value of AUC_{24h}/MIC (61) for eradication of the test organism was much lower than the widely quoted literature value of 80 h-100 h. Based on the relationship; Dose=Cl/F × AUC_{24h}/MIC × MIC_{90}, these data indicate a gentamicin once daily dosage of 1.42 mg/kg for the strain of organism used in this study (MIC=0.75 μg/mL). In Sri Lanka, Abeynayake et al. [43] reported that all the *P. multocida* isolates obtained from buffalo and cows suffering from Pasteurellosis were sensitive to gentamicin at the concentration of 1 μg/mL. Therefore, 1 μg/mL was taken as MIC_{90} of gentamicin for dosage determination, as MIC_{90} data of gentamicin against *P. Multocida* is not available in buffalo species. The corresponding estimated dose for gentamicin by taking literature value of MIC_{90} (1 μg/mL) would be 1.90 mg/kg. In broader sense, the calculated dose (2.48 mg/kg) of gentamicin based on literature recommended value of AUC_{24}/MIC for aminoglycosides is very near to the dose (1.90 mg/kg) calculated by using PK-PD modelled values of AUC_{24h}/MIC. This validates the use of PK-PD modeling approach as a basis for optimization of gentamicin

dosage for its clinical use against Pasteurellosis. The minor discrepancy in the dose might be due to the fact that literature quoted value of AUC_{24h}/MIC had been established either in laboratory animals or human patients but not in bovines or other species of farm animals. There is a need to determine these values in the target species to achieve maximum accuracy and efficacy of dose regimens of antimicrobials. The gentamicin estimated dose by taking literature MIC_{90}=4 μg/mL would be 7.56 mg/kg using PK-PD modeled AUC_{24h}/MIC value of 61 h [33]. Overall, the present findings support the use of low dose of 2-2.5 mg/kg of gentamicin in buffalo species for achieving optimum clinical and bacteriological cure for Pasteurellosis and may help in reducing the risk of development of toxicity. This is the first report in buffalo species where PK-PD modeling approach along with mutant selective window (MSW) concept is used for dosage optimization of gentamicin. Nevertheless, the MIC of antimicrobials is influenced by the inoculum size, or the size of the bacterial population used for its determination. Our MIC data for gentamicin pertains to 4-6×10^6 cfu/mL inoculums size of *P. multocida*. In acute infections, inoculums are usually of $\geq 1 \times 10^8$ cfu/mL, MIC may be escalated in such situations. Hence the higher dose may be required to treat severe infections. Therefore, determination of MICs of antimicrobials with varying levels of inoculums size of pathogens would be our future research interest.

Conclusions

It is concluded that the MPC measurements along with MIC values of gentamicin allowed us to define the MSW for *P. multocida* in buffalo calves. Taking into consideration MPC values of gentamicin along with PK-PD indices it seems that likelihood for selection of resistant mutants will be minimal at the dose rate of 2.5 mg/kg in buffalo calves. However, further studies are warranted to establish *in vitro* MIC and MPC data for large number of *P. multocida* isolates to generate MIC_{90} and MPC_{90} data that will be useful in optimizing the dosage regimens for prevention of resistant emergence. Using PK-PD modeling and integration, it is suggested that gentamicin at the dose rate of 2-2.5 mg/kg would be effective in treating infections in buffalo calves caused by *P. multocida* with MIC_{90} of ≤ 1.0 μg/mL. However, in worst clinical infections associated with *P. multocida* of $MIC_{90} \geq 4.0$ μg/mL, a higher dose at the rate of 7.5 mg/kg is recommended.

Competing Interests

There are no competing interests.

Authors Contributions

PKS was the team leader in designing and executing the study and wrote the manuscript. G Kaur, G Kumbhar and BRM performed the laboratory work, analyzed the data for the PK-PD modeling and assisted in writing. MPG provided valuable expert advice in designing the experiments, provided laboratory facilities to conduct this work and assisted in writing.

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